REMARKS

I. PTO Form 1449

Applicants respectfully request the Examiner to return an initialed and signed copy of the list of references filed with the Information Disclosure Statement in this application on May 30, 2003.

II. Claim Status

Claims 1, 3, 10, 13, 16, 19, 22, 25 and 31-62 are all the claims pending in the application. Claims 31-62 have been withdrawn from consideration. Claims 1, 3, 10, 13, 16, 19, 22 and 25 have been rejected. No claims are allowed.

Although the Examiner did not include claim 62 in the list of pending claims, Applicants note that this claim has not been canceled.

After entry of this amendment, claims 1, 3, 10, 13, 16, 19, 22, 25, 31-62 and 70 will be all the claims pending in the application.

III. Amendments to the Claims

Claim 1 has been amended to recite a fragment of mutant BAD, wherein the fragment comprises the BH3 domain of mutant BAD and has cell death promoting activity *in vitro*. Support for this amendment can be found at page 9 of the specification, which describes fragments comprising a domain substantially identical to the BH3 domain of SEQ ID NO:1,

wherein the serine at position 118 is substituted. In addition, at pages 76-77 of the specification the isolated BAD BH3 domain is shown to bind Bcl-X_L.

Claim 3 has been amended to recite a mutant BAD identical to SEQ ID NO:1 with the proviso that the amino acid "at position 118," rather than the amino acid "at the position corresponding to position 118," is alanine or an amino acid conservative for alanine.

Claim 13 has been canceled.

Claim 70, reciting the mutant BAD or fragment of mutant BAD of claim 16, wherein said alanine enhances the *in vitro* cell death promoting activity of said mutant BAD or fragment of mutant BAD, has been added. Support for this amendment can be found in Figure 11 and at page 87 of the specification, which describe BAD mutants with enhanced apoptotic activity.

I. Rejection of Claims Under 35 U.S.C. § 112, First Paragraph

At page 2 of the Office Action, claims 1, 3, 10, 13, 16, 19, 22 and 25, all of the claims currently under examination, were rejected under 35 U.S.C. § 112, first paragraph, as being non-enabled.

The Examiner stated that the specification is enabling for a mutant BAD having the amino acid sequence of SEQ ID NO:1, wherein the serine at position 118 is replaced with alanine or a conservative amino acid of alanine, and said mutant BAD has cell death promoting activity *in vitro.*

However, the Examiner contended that the specification is not enabling for the following aspects of the claimed invention:

1. A mutant BAD having an amino acid sequence 95% homologous to SEQ ID NO:1, wherein the residue corresponding to position 118 is replaced with alanine or a conservative amino acid of alanine, and said mutant BAD has cell death promoting activity in vitro;

2. A fragment of said mutant BAD, wherein said fragment has cell death promoting activity in vitro;

3. A mutant BAD or fragment thereof, wherein said mutant or fragment binds Bcl-XL or Bcl-2, or both, through a domain that is at least 75% homologous to a BH3 domain of a naturally-occurring or wildtype mammalian BAD; and

4. A mutant BAD or fragment thereof comprising an amino acid sequence corresponding to positions 103-123 of SEQ ID NO:1, wherein the amino acid residue at the position corresponding to position 118 of SEQ ID NO:1 is alanine or an amino acid conservative of alanine, and wherein said mutant BAD or fragment has cell death promoting activity in vitro.

A. Homologues of SEQ ID NO:1

At pages 6 and 7 of the Office Action, the Examiner stated that the difficulty of determining which amino acids can be modified with a reasonable expectation of maintaining function is well known in the art. According to the Examiner, the specification does not provide any guidance as to which mutations would result in a functional polypeptide. The Examiner has taken the position that, in light of the unpredictability in the art of protein engineering, mutant

BAD polypeptides having an amino acid sequence 95% homologous to SEQ ID NO:1 are not enabled.

Applicants' respectfully traverse the Examiner's position, for at least the following reasons.

First, general procedures for making synthetic polypeptide variants, and for isolating naturally-occurring variants, were well known in the art as of the filing date. Furthermore, because the structure and function of BAD and BAD homologues had been extensively analyzed, a person of ordinary skill in the art would have known how to design mutant polypeptides that would be reasonably certain to retain the recited activity. Finally, because an assay for determining cell death promoting activity *in vitro* is described at pages 99-100, the specification enables the identification of variants within the scope of the claims.

In particular, sequence alignments between various BAD polypeptides and between BAD and related proteins can be used to determine which residues are conserved and therefore likely to be essential for function. For example, Table I at page 41 of the present specification shows the human BAD of SEQ ID NO:1 aligned against the murine BAD of SEQ ID NOs: 2 and 3. In addition, several journal articles demonstrate the use of sequence alignments to indicate primary sequence homology between the critical BH3 domains of BAD and other apoptosis regulators of the BCL-2 family. See, e.g., Zha et al., *BH3 Domain of BAD is Required fro Heterodimerization with BC-X_L and Pro-apoptotic Activity*, JBC 272:24101 (1997) (Fig. 3A) and Kelekar et al., *Bad is a BH3 Domain-Containing Protein that Forms an Inactivating Dimer with Bcl-X_L*, Mol. Cell. Biol. 17:7040 (1997) (Fig. 3).

Furthermore, functional mutants of BAD can be designed by analogy to other well-studied members of the BCL-2 family. The crystal structure of BAK, for example, had been solved as of the filing date of the present application. Sattler et al., *Structure of the Bcl-X_L-Bak Complex: Recognition Between Regulators of Apoptosis*, Science 275:983 (1997) provides considerable information regarding the relationship between the structure and the function of BAK, particularly the relationship between the structure of the BH3 domain of BAK and the function of that domain. In particular, the authors test the binding affinities of numerous mutant BAK BH3-containing peptides, and demonstrate that the BAK peptide adopts an amphipathic α-helix that interacts with Bcl-X_L through hydrophobic and electrostatic interactions.

In addition, the relationship between the structure and the function of the BAD protein itself has also been investigated. Fig. 2B of Zha et al., for example, shows the predicted structure of the BH3 amphipathic α-helix of BAD, providing views of the hydrophobic and polar surfaces and indicating the likely positions of the amino acid residues. Zha et al. also use site-directed mutagenesis to substitute individual amino acids within the BH3 region of BAD, and assess the effect of the various mutations on both *in vitro* and *in vivo* heterodimerization with Bcl-X_L, and on cell death promoting activity (Figs. 5 and 6).

Thus, one of ordinary skill in the art would have been able to predict with reasonable certainty the effect that specific mutations would have on the function of BAD. Furthermore, the *in vitro* cell death promoting activity of homologues can be readily confirmed using assays well known in the art, including the apoptosis assay set forth at pages 99-100 of the specification.

In view of the above, Applicants respectfully request reconsideration and withdrawal of this aspect of the enablement rejection.

B. "Position corresponding to"

At page 5 of the Office Action, the Examiner contended that there is no definition of "corresponding," so that the phrase "an amino acid position *corresponding* to position 118 of SEQ ID NO:1" (see claim 1) indicates an amino acid at any position. Similarly, the Examiner stated that an amino acid sequence corresponding to positions 103-123 of SEQ ID NO:1 could be any amino acid fragment of any structure.

Claim 3, which does not encompass homologues that may have substitutions or deletions, has been amended to recite "an amino acid substitution at position 118 of SEQ ID NO: 1" rather than "an amino acid substitution at the position corresponding to position 118 of SEQ ID NO:1." Accordingly, this aspect of the enablement rejection is moot with regard to claim 3.

With respect to claims 1, 10, 13, 16, 19, 22 and 25, which do encompass homologues that may have substitutions or deletions, Applicants respectfully submit that a person of ordinary skill in the art would recognize that "an amino acid position corresponding to position 118 of SEQ ID NO:1" indicates only a single and specific position within the well-characterized BH3 domain of BAD. On page 45, for example, the specification explains that sequence alignment allows identification of regions of sequence homology, in particular the identification of the position corresponding to the serine at position 118 of SEQ ID NO:1. In addition, Table 1 at page 41 illustrates how the position corresponding to 118 can be identified using sequence alignment.

Similarly, a person of ordinary skill in the art would know how to determine the amino acid sequence corresponding to positions 103-123 of SEQ ID NO:1.

Thus, Applicants respectfully request reconsideration and withdrawal of this aspect of the enablement rejection.

C. Fragments

At pages 8 and 9, the Examiner contended that the specification does not teach how to make fragments of mutant BAD that would function as claimed. The Examiner stated that although the BH3 domain is necessary for interacting with Bcl-2, it is unpredictable whether the BH3 domain alone is sufficient for promoting cell death activity, or whether other parts of SEQ ID NO:1 might be required. The Examiner concluded that screening for functional fragments would entail undue experimentation.

Claim 1 has been amended to recite fragments comprising the BH3 domain of mutant BAD and having cell death promoting activity. Applicants submit that a person of ordinary skill in the art would know how to make and use the recited fragments.

First, the BH3 domain of BAK is sufficient not only for interacting with Bcl-XL but also for promoting apoptosis. Thus, based on both functional and structural similarities between the BH3 domain of BAD and the BH3 domain of BAK, a person of ordinary skill in the art would reasonably expect the BH3 domain of SEQ ID NO: 1 to be sufficient for promoting cell death activity. Chittenden et al., *A Conserved Domain in Bak, distinct from BH1 and BH2, Mediates Cell Death and Protein Binding Functions*, EMBO J. 14:5589 (1995), states that "[w]e have

identified a domain in BAK that is both necessary and sufficient for cytotoxic activity and binding to Bcl-X_L (abstract)." In addition, at page 983 Sattler et al. notes that "only a relatively small portion of the death-promoting proteins encompassing the BH3 region is critical for the ability to promote apoptosis . . . small truncated forms of Bak are necessary and sufficient both for promoting cell death and binding to Bcl-X_L".

Furthermore, studies of the BAD peptide directly indicated that the BH3 domain of BAD is not only necessary but also sufficient for function. Kelekar et al., for example, found that the BH3 domain of BAD is both necessary and sufficient to bind Bcl-X_L (abstract). Similarly, Zha et al. show that the BAD BH3 domain is both sufficient and essential for BAD to heterodimerize with BCL-2 (page 24102). The pro-apoptotic activity of BAD is due to heterodimerization with Bcl-X_L. Accordingly, a skilled artisan would reasonably expect a peptide that binds Bcl-X_L to also promote cell death activity. In this regard, Zha et al. note that the death agonists BIK and BID possess only the BH3 domain, and suggest that the BH3 domain represents the minimal death domain (page 24104). Finally, recent studies have shown that isolated BAD BH3 peptides do in fact demonstrate pro-apoptotic activity when introduced into cells. See, e.g., Letai et al., *Distinct BH3 Domains Either Sensitize or Activate Mitochondrial Apoptosis, Serving as Prototype Cancer Therapeutics*, Cancer Cell 2:183 (2002).

For at least the foregoing reasons, Applicants respectfully request reconsideration and withdrawal of this aspect of the enablement requirement.

D. Polypeptides with 75% homologous BH3 domains

At page 9 of the Office Action, the Examiner contended that Applicants have not taught how to make functional BAD mutants or fragments that bind Bcl-X_L and/or Bcl-2 through a domain that is at least 75% homologous to a BH3 domain of a naturally occurring or wild-type mammalian BAD.

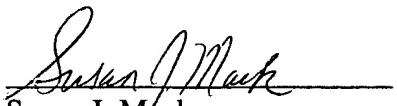
Claim 13 has been canceled, rendering this aspect of the enablement rejection moot.

IV. Conclusion

In view of the above, reconsideration and allowance of this application are now believed to be in order, and such actions are hereby solicited. If any points remain in issue which the Examiner feels may be best resolved through a personal or telephone interview, the Examiner is kindly requested to contact the undersigned at the telephone number listed below.

The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

Respectfully submitted,



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16. The protein is predicted to be largely helical [B. Rost and C. Sander, *Proteins* **19**, 55 (1994)], but prediction-based threading [B. Rost, in *Protein Folds, A Distance-Based Approach*, H. Bohr and S. Brunak, Eds. (CRC Press, Boca Raton, FL, 1995), pp. 132–151; B. Rost, in *The Third International Conference on Intelligent Systems for Molecular Biology (ISMB)*, C. Rawlings *et al.*, Eds. (AAAI Press, Menlo Park, CA, 1995), pp. 314–321] fails to identify any other proteins of similar structure; http://www.embl-heidelberg.de/predictprotein/predictprotein.html.
17. T. E. Creighton, *Proteins: Structures and Molecular Properties* (Freeman, New York, ed. 2, 1993).
18. A. Schöler and H. J. Schüller, *Mol. Cell. Biol.* **14**, 3613 (1994); M. Proft, D. Grzesitza, K. D. Entian, *Mol. Gen. Genet.* **246**, 367 (1995).
19. All yeast manipulations were in the SEY6210 background (*MATa, leu2-3, ura3-52, his3-Δ200, lys2-801, trp1-Δ901, suc2-Δ9*). The *CAT5/COQ7* locus was disrupted with a PCR-mediated approach [A. Baudin, O. Ozier-Kalogeropoulos, A. Denouel, F. Lacroute, C. Cullin, *Nucleic Acids Res.* **21**, 3329 (1993); the primers used were SHP84 and SHP85]. The *CAT5/COQ7* gene was entirely replaced with a DNA fragment containing a disruption module encoding the green fluorescent protein and the *HIS3* gene [R. K. Niedenthal, L. Riles, M. Johnston, J. H. Hehemann, *Yeast* **12**, 773 (1996)]. Haploid cells were transformed [R. D. Gietz, R. H. Schiestl, A. R. Willems, R. A. Woods, *ibid.* **11**, 355 (1995)] with the PCR product, and *HIS3* integrants were selected on minimal medium lacking histidine. Gene disruptions were confirmed by PCR analysis with primers SHP82, SHP83, and ML138. The *Δcat5/coq7* strain failed to grow (*24*) on YEPG or $YEPE_3$, which contains ethanol (*11*). The sequences of the primers are available on request.
20. The *CAT5/COQ7* locus was directly amplified from yeast genomic DNA by PCR with *Pfu* polymerase (Stratagene) and primers SHP69 and SHP70. A cDNA corresponding to the entire *clk-1* coding sequence was obtained by PCR amplification, also with *Pfu* polymerase, and nested primer pairs SHP57 and SHP59 and then SHP57 and SHP58 on single-stranded cDNA that had been synthesized by priming with SHP59. The respective yeast and nematode PCR products were digested with Hind III and ligated to Hind III–cut and dephosphorylated pVT102-U [T. Vernet, D. Dignard, D. Y. Thomas, *Gene* 52, 225 (1987)]. As well as restoring growth on glycerol, both the *CAT5/COQ7*- and *clk-1*–containing plasmids restored the ability of the *Δcat5/coq7* strain to grow on ethanol [$YEPE_3$ medium (*24*)]. The *Δcat5/coq7* strain transformed with the *CAT5/COQ7*-containing plasmid did not grow as well as the wild-type yeast strain on nonfermentable carbon sources. When the yeast gene was reintroduced in the context of its own promoter on a centromeric vector, full restoration of wild-type growth was obtained (*24*). *CAT5/COQ7* is known to be involved in the regulation of its own expression (*11*); presumably, the presence of excess Cat5p/Coq7p perturbs the normal metabolic balance of yeast. The sequences of the primers are available on request.
21. E. W. Jones, J. R. Pringle, J. R. Broach, Eds., *The Molecular and Cellular Biology of the Yeast* Saccharomyces (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1992).
22. J. Campisi, *Cell* **84**, 497 (1996); L. Guarente, *ibid.* **86**, 9 (1996).
23. T. M. Barnes, Y. Jin, H. R. Horvitz, G. Ruvkun, S. Hekimi, *J. Neurochem.* **67**, 46 (1996).
24. B. Lakowski and J. Ewbank, unpublished data.
25. By picking Sma non-Dpy recombinant progeny of *dpy-17(e164) sma-4(e729)/unc-79(e1030) clk-1(e2519) lon-1(e185)* hermaphrodites, we were able to position *clk-1* more precisely: *dpy-17* 25/79 *clk-1* 27/79 *lon-1* 27/79 *sma-4*. To interpolate the physical position of *clk-1*, we estimated that the separation between *ced-4* and *dpy-17* is ~0.2 centimorgans on the basis of data in the database ACeDB (*7*). By using linked double mutants, we also directly determined (*24*) the two point distances between *dpy-17* and *sma-4* (0.85 cM), *dpy-17* and *lon-1* (0.5 cM), and *lon-1* and *sma-4* (0.35 cM). Details of the mapping data can be found in ACeDB (*7*).
26. The sequencing of allele *qm11*, which has a phenotype essentially identical to *e2519* (*1*), revealed an identical lesion. The low probability of independently obtaining the same mutation twice suggests that the original allele was lost. Sequencing of *qm47* failed to reveal a mutation. Subsequent reexamination of the phenotype of *qm47* homozygotes and new complementation tests suggest that *qm47* is not a *clk-1* allele.
27. We thank A. Coulson for cosmids; K. Kemphues for strains; J.-C. Labbé, A Kothari, and J. Mes-Mason for nematode, mouse, and human RNA, respectively; and A. Wong, A.-M. Sdicu, and R. Durbin. Some nematode strains used in this work were provided by the Caenorhabditis Genetics Center, which is funded by the NIH National Center for Research Resources. Supported by a Royal Society–National Science and Engineering Research Council of Canada exchange fellowship and a Medical Research Council of Canada fellowship to J.J.E., a Medical Research Council of Canada grant to S.H., a Canadian Genome Analysis and Technology grant to H.B., and by fellowships to B.L. from the J. W. McConnell Foundation and Fonds pour la Formation de Chercheurs et l'Aide à la Recherche Québec.

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Structure of Bcl-x_L–Bak Peptide Complex: Recognition Between Regulators of Apoptosis

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Heterodimerization between members of the Bcl-2 family of proteins is a key event in the regulation of programmed cell death. The molecular basis for heterodimer formation was investigated by determination of the solution structure of a complex between the survival protein Bcl-x_L and the death-promoting region of the Bcl-2–related protein Bak. The structure and binding affinities of mutant Bak peptides indicate that the Bak peptide adopts an amphipathic α helix that interacts with Bcl-x_L through hydrophobic and electrostatic interactions. Mutations in full-length Bak that disrupt either type of interaction inhibit the ability of Bak to heterodimerize with Bcl-x_L.

Programmed cell death (apoptosis) occurs during the course of several physiological processes, and when dysregulated contributes to many diseases, including cancer, autoimmunity, and neurodegenerative disorders (*1*). The Bcl-2 family of proteins plays a central role in the regulation of apoptotic cell death induced by a wide variety of stimuli (*2*). Some proteins within this family, including Bcl-2 and Bcl-x_L, inhibit programmed cell death, and others, such as Bax and Bak, can promote apoptosis. Interactions between these two groups of proteins antagonize their different functions and modulate the sensitivity of a cell to apoptosis (*3*, *4*). Several regions of the death-inhibiting proteins participate in their antiapoptotic activity and heterodimerization with the death-promoting proteins, including the Bcl-2 homology 1 (BH1) and BH2 regions (*3*, *5*, *6*). In contrast, only a relatively small portion of the death-promoting proteins encompassing the BH3 region is critical for the ability to promote apoptosis (*7*– *10*). For example, small, truncated forms of Bak are necessary and sufficient both for promoting cell death and binding to Bcl-x_L (*7*).

The three-dimensional (3D) structure of the cell survival protein Bcl-x_L consists of two central hydrophobic α helices surrounded by five amphipathic helices (*11*). To understand how Bak interacts with Bcl-x_L and inhibits the ability of Bcl-x_L to promote cell survival, we determined the solution structure of Bcl-x_L complexed with a 16-residue peptide derived from the BH3 region of Bak. We also measured the binding affinities of Bcl-x_L to alanine mutant Bak peptides and to peptides corresponding to the BH3 regions of other Bcl-2 family members (*12*, *13*).

The minimal region of Bak required to bind to Bcl-x_L was examined in a fluorescence-based assay (*14*). A 16–amino acid peptide derived from the BH3 region of Bak (residues 72 to 87) bound tightly to Bcl-x_L (Table 1). In contrast, smaller peptides from this region, such as an 11–amino acid peptide corresponding to residues 77 to 87, did not bind (Table 1). The 16–amino acid peptide of Bak corresponds precisely to the

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region of Bcl-x_L that forms the second α helix (*11*).

The structure of the 16–amino acid peptide (*15*) complexed with a biologically active deletion mutant of Bcl-x_L (*16*) was determined by nuclear magnetic resonance spectroscopy (NMR). The structure was determined from a total of 2813 NMR-derived restraints and is well defined by the NMR data (Fig. 1A) (*17*). The atomic root-mean-square deviation (rmsd) about the mean coordinate positions for residues 1 to 205 of Bcl-x_L and 72 to 87 of the Bak peptide was 0.79 ± 0.15 Å for the backbone and 1.21 ± 0.13 Å for all heavy atoms.

Overall, the structure of the truncated form of Bcl-x_L when complexed to the Bak peptide is similar to the x-ray and NMR structures of uncomplexed Bcl-x_L (*11*, *18*). The Bak peptide binds in a hydrophobic cleft formed by the BH1, BH2, and BH3 regions of Bcl-x_L (Figs. 1 and 2). Although a random coil when free in solution (*19*), the Bak peptide forms an α helix when complexed to Bcl-x_L. The NH_2-terminal residues of the peptide show numerous nuclear Overhauser effects (NOEs) to residues in the BH1 region of Bcl-x_L (Val^{126}, Glu^{129}, Leu^{130}, and Phe^{146}), whereas the COOH-terminal portion of the Bak peptide interacts predominantly with residues in the BH2 and BH3 regions (Phe^{97}, Arg^{100}, Tyr^{101}, and Phe^{105}). The hydrophobic side chains of the peptide (Val^{74}, Leu^{78}, Ile^{81}, and Ile^{85}) point into a hydrophobic cleft of Bcl-x_L (Fig. 2) and stabilize complex formation. In addition to these hydrophobic interactions, the charged side chains of the Bak peptide (Arg^{76}, Asp^{83}, and Asp^{84}) are close to oppositely charged residues of Bcl-x_L (Glu^{129}, Arg^{139}, and Arg^{100}, respectively) (Fig. 2).

To identify the interactions that are important for complex formation, we measured the binding affinities of mutant Bak peptides containing alanine substitutions (Table 1) (*14*). A decrease in binding affinity by a factor of 800 was observed for the Bak peptide in which Leu^{78} is substituted by an alanine. This can be explained by the loss of extensive interactions between the side chain of Leu^{78} of Bak and the hydrophobic pocket formed by Tyr^{101}, Leu^{108}, Val^{126}, and Phe^{146} of Bcl-x_L (Fig. 2B). Mutation of other hydrophobic residues of Bak (Ile^{85}, Ile^{81}, and Val^{74}) to alanine also resulted in reduced binding to Bcl-x_L (Table 1), which further demonstrates the importance of hydrophobic interactions in complex formation. The hydrophobic residues at these positions are largely conserved in the Bcl-2 family of proteins (Table 1). In contrast, Ile^{80} is not conserved and is located on the surface of the complex (Fig. 2), consistent with the negligible loss in binding affinity observed when this residue was changed to an alanine.

Analysis of the structure (Fig. 2) suggested that the interaction between Asp^{83} of the Bak peptide and Arg^{139} of Bcl-x_L would stabilize complex formation. Indeed, Asp^{83} is completely conserved within the Bcl-2 family of proteins, and when substituted with alanine in the Bak peptide, markedly reduced the binding of this peptide to Bcl-x_L (Table 1). Moreover, Arg^{139} is highly conserved, and mutation of Arg^{139} to Gln in Bcl-x_L inhibits its antiapoptotic activity and binding to the Bax protein (*20*). It was also expected from the structure (Fig. 2) that electrostatic interactions between Arg^{76} of Bak and Glu^{129} of Bcl-x_L would contribute to complex formation. This is supported by the observed decrease in binding to Bcl-x_L of a Bak peptide in which Arg^{76} is mutated to alanine (Table 1).

Table 1. Binding affinities (*14*) of peptides to Bcl-x_L. Residues of Bak peptide substituted with alanine are in boldface. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

Peptide	Sequence		K_D (μM)
Bak	72 GQVGRQLAIIGDDINRRYDSEFQ	94	0.20 ± 0.02
	72 GQVGRQLAIIGDDINR.....	87	0.34 ± 0.03
	77 .....QLAIIGDDINR.....	87	No binding
	GQ**A**GRQLAIIGDDINR.....		15 ± 3
	GQVG**A**QLAIIGDDINR.....		3.3 ± 1
	GQVGRQ**AA**IIGDDINR.....		270 ± 90
	GQVGRQLA**A**IGDDINR.....		1.0 ± 0.2
	GQVGRQLAI**A**GDDINR.....		17 ± 6
	GQVGRQLAII**A**DDINR.....		0.50 ± 0.1
	GQVGRQLAIIG**A**DINR.....		41 ± 4
	GQVGRQLAIIGD**A**INR.....		0.14 ± 0.02
	GQVGRQLAIIGDD**A**NR.....		93 ± 20
Bcl-2	91 PVVHLALRQAGDDFSR.....	106	6.4 ± 0.8
Bax	57 KKLSECLKRIGDELDS.....	72	13 ± 3
Bik	55 DALALRLACIGDEMDV.....	70	15 ± 6
Bcl-x_L	84 AAVKQALREAGDEFEL.....	99	325

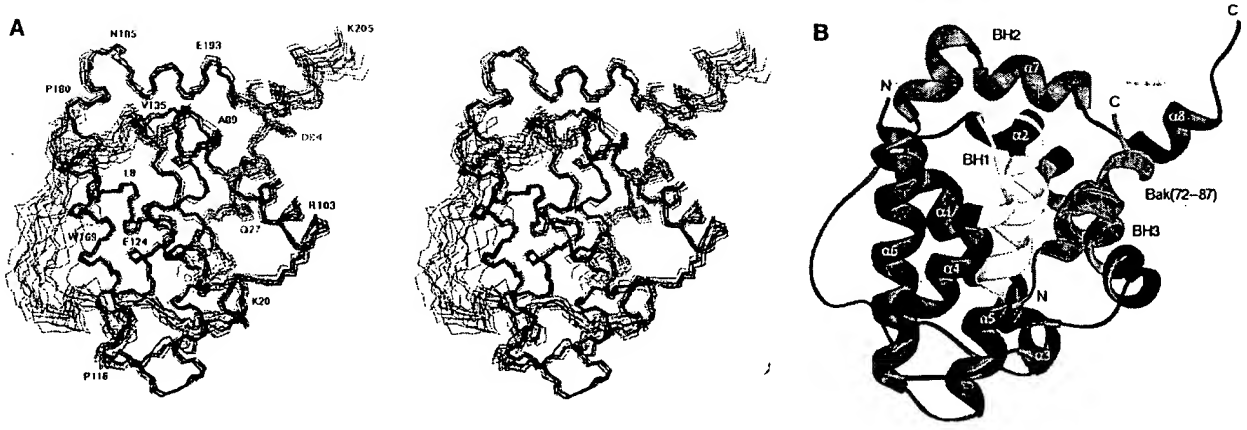

Fig. 1. (**A**) Stereoview of the backbone (N, C^{α}, C′) of 15 superimposed NMR-derived structures of Bcl-x_L (shown in black) complexed with the 16–amino acid Bak peptide (shown in red). (**B**) Ribbons (*21*) depiction of the averaged minimized NMR structure for the complex. The BH1, BH2, and BH3 regions of Bcl-x_L are shown in yellow, red, and green, respectively. The Bak peptide is shown in magenta.

However, the potential charge-charge interaction between Asp^{84} of Bak and Arg^{100} of Bcl-x_L does not appear to be critical for complex stabilization as a negligible effect on binding to Bcl-x_L was observed when Asp^{84} was substituted by an alanine (Table 1).

Interactions within the Bcl-2 family of proteins exhibit a defined selectivity and hierarchy (*12*, *13*). To investigate whether this selectivity is conferred by the BH3 regions from other Bcl-2 family members, we measured the binding affinities of a series of BH3-containing peptides to Bcl-x_L (*14*). Subtle differences in the amino acid sequences of the BH3 regions among members of the Bcl-2 family give rise to distinct differences in the affinities of these peptides for Bcl-x_L (Table 1). The Bak peptide binds to Bcl-x_L with greater affinity than any of the other peptides, including the peptides derived from the other death-promoting proteins, Bax and Bik. The Bcl-x_L peptide binds with the weakest affinity to Bcl-x_L, consistent with the monomeric nature of this protein (*11*). The selectivity of Bcl-x_L that we observed for the peptides from different Bcl-2 family members is consistent with the selectivity for heterodimer formation amongst the Bcl-2 family of proteins and suggests that the BH3 region plays a central role in defining the binding specificity of the Bcl-2–related proteins for Bcl-x_L.

The molecular interactions that stabilize the Bcl-x_L–Bak peptide complex likely reflect the important interactions that occur between the full-length proteins. The wild-type Bak peptide can inhibit the interaction of Bcl-x_L with full-length Bak or Bax in a concentration-dependent manner (*20*). Furthermore, Bak peptides containing alanine substitutions for Leu^{78} and Asp^{83}, which markedly reduced their binding to Bcl-x_L (Table 1), were unable to block heterodimer formation between-full length Bcl-x_L and Bak (Fig. 3A). When these two residues (Leu^{78} and Asp^{83}) were mutated in the full-length Bak protein, the mutant Bak proteins failed to coprecipitate with Bcl-x_L even though they were expressed at levels comparable to that of the wild type protein (Fig. 3B). Thus, the reduction in binding to Bcl-x_L observed with the full-length mutant Bak proteins resembles the loss in binding to Bcl-x_L measured for the mutant Bak peptides. These data are consistent with previous reports (*7*–*10*) on the functional importance of the BH3 region of the death-promoting proteins. This region of Bak and similar sequences in Bax and Bik (Bip1) promote apoptosis and interact with Bcl-x_L (*7*, *8*). In addition, neither the BH1 nor the BH2 region of Bax is necessary for binding to Bcl-2 or for promoting cell death (*9*, *10*).

Using the structure of the Bcl-x_L–Bak peptide complex and a homology model of the Bak protein, we modeled the structure of the heterodimer of the full-length proteins. In the structure of Bak based on its homology to Bcl-x_L, the hydrophobic side chains of the amphipathic α2 helix containing the BH3 region point toward the interior of the Bak protein, making these residues unavailable to interact with Bcl-x_L. Thus, binding to Bcl-x_L would necessitate a conformational change in the Bak protein to expose the hydrophobic surface of α2. One possibility is a rotation of the α2 helix along the helix axis that would allow the formation of the same hydrophobic and charge-charge interactions observed in the NMR structure of the Bcl-x_L–Bak peptide complex. It is of interest that based on the structure of Bcl-x_L, this helix is predicted to be flanked by highly flexible loops on both ends that could allow such a rotation.

In summary, our structure of the Bcl-x_L–Bak peptide complex reveals the structural basis for the requirements of the BH1, BH2, and BH3 regions for heterodimer formation among Bcl-2 family members. These data suggest that the formation of a hydrophobic binding cleft and properly positioned charged residues are required for the anti-apoptotic functions of Bcl-x_L. Indeed, a variety of mutations that would be predicted to alter the accessibility or binding properties of this region in Bcl-x_L and Bcl-2, including G138A (*3*), R139Q (*20*), Y101K (*20*), and L130A (*20*), have been shown to inhibit the function of this protein. For proteins that promote cell death, only the BH3 region is required for activity (*7*–*10*), which as shown here forms an amphipathic α helix and binds with high affinity to the hydrophobic groove in Bcl-x_L. Some proteins that promote cell death—such as Bik—have homology to other Bcl-2 pro-

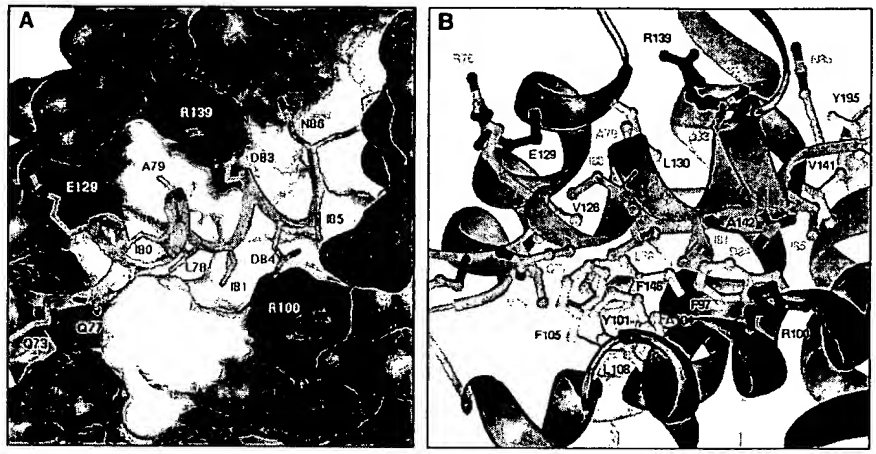

Fig. 2. (**A**) Surface representation of the binding pocket of Bcl-x_L bound to the Bak peptide. Hydrophobic residues showing NOEs to the peptide are colored in yellow, whereas Arg^{139}/Arg^{100} and Glu^{129} are colored in blue and red, respectively. Residues of Bcl-x_L are labeled in white and the Bak peptide in black. (**B**) Depiction of the side chains in the binding site of Bcl-x_L. Hydrophobic side chains of the protein showing NOEs to the peptide are colored in yellow. Side chains of positively and negatively charged side chains interacting with the peptide are colored in blue and red, respectively. The peptide side chains are colored by atom type. Residues of Bcl-x_L and the Bak peptide are labeled in black and green, respectively.

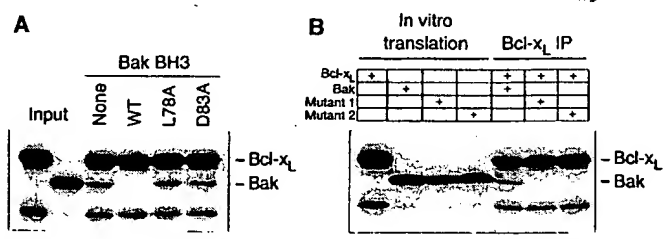

Fig. 3. (**A**) Mutations of critical residues in the Bak BH3 peptide abolish its ability to inhibit Bcl-x_L heterodimerization with Bak. In vitro–translated Bcl-x_L and Bak were combined together with 100 μM of the indicated Bak BH3 peptide. The reaction was immunoprecipitated with an antibody to Bcl-x (anti-Bcl-x), and the immunoprecipitated products were resolved by SDS–polyacrylamide gel electrophoresis (PAGE). (**B**) Mutations in Bak BH3 residues that are predicted to be involved in Bcl-x_L–Bak interactions abolish heterodimerization. In vitro–translated Bcl-x_L, Bak, or mutants of Bak were combined as indicated and immunoprecipitated with anti–Bcl-x. The immunoprecipitated products were resolved by SDS-PAGE. Bak mutation 1 contains a glutamic acid in place of arginine at amino acid 76 and an arginine in place of aspartic acid at amino acid 83. Bak mutant 2 contains an alanine in place of leucine at amino acid 78.

teins only within the BH3 region. In contrast, other Bcl-2–related proteins such as Bak or Bax are predicted to have more extensive structural similarities to Bcl-x_L. For these proteins, our studies suggest that a structural change may be required for the BH3 region to participate in dimerization.

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14. The binding affinities of peptides to full-length Bcl-x_L were measured from the fluorescence emission of the Trp residues of Bcl-x_L as a function of increasing peptide concentration. The excitation and emission wavelengths were 290 and 340 nm, respectively.
15. Unlabeled peptide (GQVGRQLAIIGDDINR) and a peptide uniformly ^{15}N-,^{13}C-enriched for the Gly, Ala, Val, Leu, and Ile residues were purchased from PeptidoGenic Research (Livermore, CA) and purified by reversed-phase high-performance liquid chromatography on a C8 column. NMR samples (1 to 3 mM) of a 1:1 protein-peptide complex were prepared in a 10 mM sodium phosphate buffer (pH 6.5) in ^2H_2O or a 9:1 mixture of H_2O and ^2H_2O.
16. The deletion mutant of Bcl-x_L used in the NMR studies lacks the putative COOH-terminal transmembrane region and residues 45 to 84, which constitute a flexible loop previously shown to be dispensible for the antiapoptotic activity of Bcl-x_L (*11*). The deletion mutant of Bcl-x_L was constructed from the expression vector for Bcl-x_L (residues 1 to 209) (*11*) by a procedure similar to that of M. P. Weiner *et al.* [*Gene* **151**, 119 (1994)]. Residue numbers correspond to full-length Bcl-x_L. Thus, in the Δ(45–84)Bcl-x_L construct used in this study, residues 44 and 85 are sequential. The Δ(45–84)Bcl-x_L construct also has four additional NH_2-terminal residues (numbers −3 to 0) due to cloning artifacts. We prepared uniformly ^{15}N- and ^{15}N-,^{13}C-labeled proteins by growing the *Escherichia coli* strain HMS174(DE3) overexpressing Bcl-x_L on a minimal medium containing $^{15}NH_4Cl$ with or without [U-^{13}C]glucose. We prepared uniformly ^{15}N-,^{13}C-labeled and fractionally deuterated protein by growing the cells in 75% ^2H_2O. The recombinant protein was purified by affinity chromatography on a nickel-IDA column (Invitrogen) followed by ion-exchange chromatography on an S-Sepharose column.
17. NMR spectra were acquired at 30°C on a Bruker DMX500 or AMX600 NMR spectrometer. The ^1H, ^{13}C, and ^{15}N resonances of the backbone and side chains were obtained with a sample containing the (U-^{15}N-,^{13}C)-labeled and 75% deuterated protein as described [T. Yamazaki, W. Lee, S. H. Arrowsmith, D. R. Muhandiram, L. E. Kay, *J. Am. Chem. Soc.* **116**, 11655 (1994); G. M. Clore and A. M. Gronenborn, *Methods Enzymol.* **239**, 349 (1994)]. The methyl groups of Val and Leu residues were stereospecifically assigned [D. Neri, T. Szyperski, G. Otting, H. Senn, K. Wüthrich, *Biochemistry* **28**, 7510 (1989)]. Distance restraints were obtained from ^{15}N- or ^{13}C-resolved 3D NOE spectra, and φ dihedral angle restraints were measured from $^3J_{H^N,H^\alpha}$ coupling constants [H. Kuboniwa, S. Grzesiek, F. Delaglio, A. Bax *J. Biomol. NMR* **4**, 871 (1994)]. To assign the NMR resonances of the peptide and obtain intra- and intermolecular distance restraints, we acquired 2D and 3D ^{15}N-,^{13}C-filtered experiments on a sample with (U-^{15}N-,^{13}C)-labeled protein and unlabeled peptide. Additional distance restraints from ^{15}N- and ^{13}C-separated NOE experiments were obtained with a sample of unlabeled protein complexed to the Bak peptide uniformly ^{15}N- and ^{13}C- labeled for Gly, Ala, Val, Leu, and Ile. The structure calculations were based on a distance geometry and simulated annealing protocol [J. Kuszewski, M. Nilges, A. T. Brünger *J. Biomol. NMR* **2**, 33 (1992)] with the program X-PLOR [A. T. Brünger, *X-PLOR Version 3.1*, Yale University, New Haven, CT (1992)]. NOE-derived distance restraints with a square-well potential (F_{noe} = 50 kcal mol^{-1} Å^{-2}) were used after each was categorized as strong (1.8 to 3.0 Å), medium (1.8 to 4.0 Å), or weak (1.8 to 5.0 Å) on the basis of the NOE intensity. An additional 138 distance restraints were included for 69 hydrogen bonds identified from the slowly exchanging amides and given bounds of 1.8 to 2.3 Å (H-O) and 2.8 to 3.3 Å (N-O). No distance restraint was violated by more than 0.35 Å in any of the final structures. For the ensemble, the residual NOE rmsd was 0.009 ± 0.003 Å and the E_{noe} was 15 ± 3 kcal mol^{-1}. Torsional restraints were applied to 71 φ angles (including five for the peptide) with values of −60 ± 40° (F_{cdih} = 200 kcal mol^{-1} rad^{-2}) for $^3J(H^N,H^\alpha)$ for coupling constants <5.8 Hz in α-helical regions. No torsional angle restraint was violated by more than 5° in any of the final structures. For the ensemble, the residual torsional rmsd was 0.11 ± 0.06° and the E_{cdih} was 0.1 ± 0.0 kcal mol^{-1}. The covalent geometries were well satisfied as indicated by a small total energy (137 ± 10 kcal mol^{-1}). Although the Lennard-Jones potential was not used during any refinement stage, the final structures exhibited good van der Waals geometries as illustrated by an $E_{L\text{-}J}$ of −1104 ± 12 kcal mol^{-1}.
18. The rmsd between the NMR structures of free and complexed Bcl-x_L for the C^α atoms within the common regular elements of secondary structure is 1.7 Å. When complexed to the Bak peptide, residues 101 to 103 form an extension of the second α helix, the third helix in Bcl-x_L is reduced to a single helical turn, and residues 198 to 205 form an additional helix.
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A Protein-Counting Mechanism for Telomere Length Regulation in Yeast

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In the yeast *Saccharomyces cerevisiae*, telomere elongation is negatively regulated by the telomere repeat–binding protein Rap1p, such that a narrow length distribution of telomere repeat tracts is observed. This length regulation was shown to function independently of the orientation of the telomere repeats. The number of repeats at an individual telomere was reduced when hybrid proteins containing the Rap1p carboxyl terminus were targeted there by a heterologous DNA-binding domain. The extent of this telomere tract shortening was proportional to the number of targeted molecules, consistent with a feedback mechanism of telomere length regulation that can discriminate the precise number of Rap1p molecules bound to the chromosome end.

Telomeres, the ends of linear eukaryotic chromosomes, are essential structures formed by specific protein-DNA complexes that protect chromosomal termini from degradation and fusion (*1*). One of the essential functions of telomeres is to allow the complete replication of chromosome ends, which cannot be accomplished by known DNA polymerases (*2*). The progressive loss of DNA that would occur after each round of replication is balanced by a ribonucleoprotein terminal transferase enzyme called telomerase, which specifically extends the 3′ G-rich telomeric strand in an RNA-templated reaction (*3*). In most organisms, telomeric DNA consists of a tandem array of short repeats. In yeast, the telomeric DNA is organized in a nonnucleosomal structure based on an array of the telomere repeat–binding protein Rap1p (*4*, *5*).

In the human germline, cells express telomerase and maintain a constant average telomere length. This initial size appears to determine the replicative life-span of somatic cells, in which telomerase activity is usually undetectable and telomere repeats are progressively lost at each cell division (*6*). In unicellular organisms like *S. cerevisiae*, telomere length is kept within a narrow size distribution, specific for a given strain,

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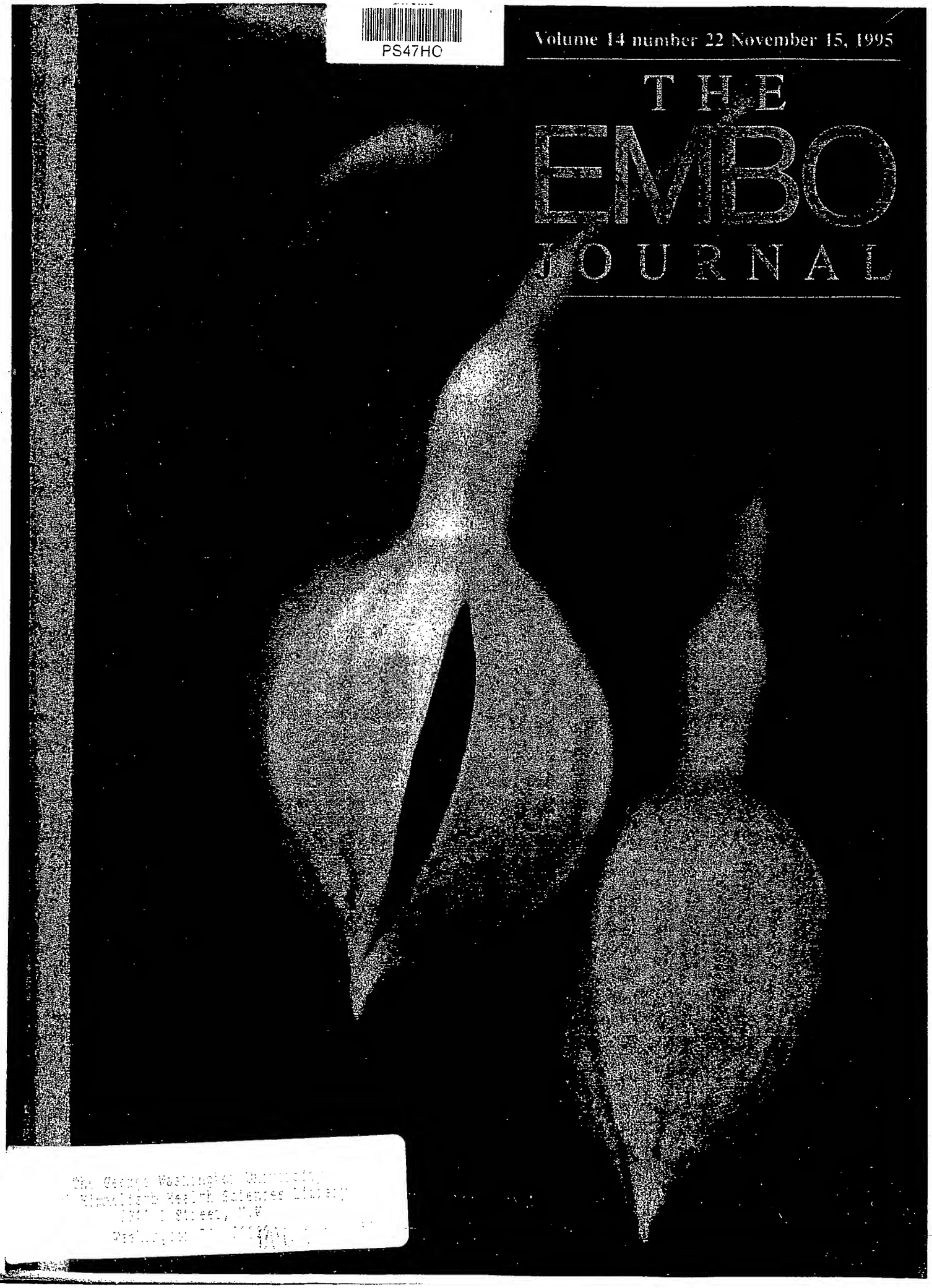

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A conserved domain in Bak, distinct from BH1 and BH2, mediates cell death and protein binding functions

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Regulation of the cell death program involves physical interactions between different members of the Bcl-2 family that either promote or suppress apoptosis. The Bcl-2 homolog, Bak, promotes apoptosis and binds anti-apoptotic family members including Bcl-2 and Bcl-x_L. We have identified a domain in Bak that is both necessary and sufficient for cytotoxic activity and binding to Bcl-x_L. Sequences similar to this domain were identified in Bax and Bip1, two other proteins that promote apoptosis and interact with Bcl-x_L, and were likewise critical for their capacity to kill cells and bind Bcl-x_L. Thus, the domain is of central importance in mediating the function of multiple cell death-regulatory proteins that interact with Bcl-2 family members.

Keywords: apoptosis/Bcl-2 homolog/Bik/programmed cell death

Introduction

Disruption of apoptosis, the intrinsic cell death program, may contribute to the onset and/or evolution of human tumors (Williams, 1991; Fisher, 1994). A particularly well-characterized genetic lesion in the apoptotic pathway is the t(14:18) translocation that activates expression of the *bcl-2* oncogene in human B cell follicular lymphomas (Bakhshi *et al.*, 1985; Cleary and Sklar, 1985; Tsujimoto *et al.*, 1985). Bcl-2 functions as a potent suppressor of cell death and can suppress apoptosis triggered by diverse stimuli (reviewed by Reed, 1994). There is also a remarkable degree of conservation of Bcl-2 function across species, suggesting that Bcl-2 is intimately connected with an evolutionarily conserved cell death pathway (Vaux *et al.*, 1991, 1994; Hengartner and Horvitz, 1994).

Bcl-2 is a member of an emerging family of proteins defined by the conservation of two domains, referred to as Bcl-2 homology domains 1 and 2 (BH1 and BH2) (Williams and Smith, 1993; Oltvai and Korsmeyer, 1994). Of the known Bcl-2 homologs, Bcl-x_L bears the highest degree of homology to Bcl-2 and, like Bcl-2, functions to suppress apoptosis (Boise *et al.*, 1993). In contrast, a second Bcl-2 homolog, Bax, forms heterodimers with Bcl-2 and Bcl-x_L and was shown to counteract Bcl-2 function and accelerate apoptosis (Oltvai *et al.*, 1993). The family of Bcl-2 relatives includes proteins from DNA viruses: Epstein–Barr virus BHRF-1 and African swine fever virus LMW5-HL (Pearson *et al.*, 1987; Neilan *et al.*, 1993). The adenovirus E1B 19 kDa protein also functions as a strong suppressor of apoptosis under diverse conditions (Rao *et al.*, 1992; White *et al.*, 1992; Subramanian *et al.*, 1993). Although E1B 19 kDa lacks sequences resembling the canonical BH1 and BH2 elements that define the Bcl-2 family, it may function in a manner similar to Bcl-2, since both proteins interact with a common set of cellular proteins (Boyd *et al.*, 1994). Thus, several viruses have independently evolved to encode structural and/or functional homologs of Bcl-2.

A complex array of protein–protein interactions among different Bcl-2 family members contributes to the regulation of the cell death program (Oltvai and Korsmeyer, 1994). The anti-apoptotic activity of Bcl-2, for example, may be dependent upon its interaction with death-promoting proteins such as Bax. Mutations in Bcl-2 that eliminate heterodimerization with Bax, abrogate the ability of Bcl-2 to suppress cell death (Yin *et al.*, 1994). A combinatorial interplay between interacting proteins may also play a significant regulatory role. It was suggested that the interaction of Bad, a Bcl-2-related protein, with Bcl-x_L, promotes cell death due to the competitive displacement of Bax from Bcl-x_L (Yang *et al.*, 1995).

We and others recently described a Bcl-2 relative, Bak, that opposes Bcl-2 function and triggers apoptosis under certain conditions (Chittenden *et al.*, 1995; Farrow *et al.*, 1995; Kiefer *et al.*, 1995). Bak interacts with both Bcl-2 and (more strongly) Bcl-x_L (Chittenden *et al.*, 1995), as well as E1B 19 kDa (Farrow *et al.*, 1995). These data have raised the possibility that proteins like Bcl-x_L, Bcl-2 and E1B 19 kDa that suppress apoptosis may operate by binding and inactivating Bak, and/or other death-promoting proteins such as Bax.

In view of the importance of these interactions for the regulation of cell death, we sought to understand in greater detail the molecular requirements for a prototypical interaction involving a cell death promoter, Bak, with a cell death suppressor, Bcl-x_L. We report here the identification of a domain in the Bak protein, distinct from BH1 and BH2, that is uniquely required for both its cell killing and Bcl-x_L binding functions, and is itself sufficient for these activities. Moreover, sequences homologous to this domain were identified in Bax, and in an otherwise unrelated protein, Bip1 (Boyd *et al.*, 1995), that also promotes cell death and binds to Bcl-x_L. The homologous sequence motifs were shown to be important for the cell killing and protein binding functions of these proteins, indicating that this domain is critical to the actions of multiple cell death-promoting proteins.

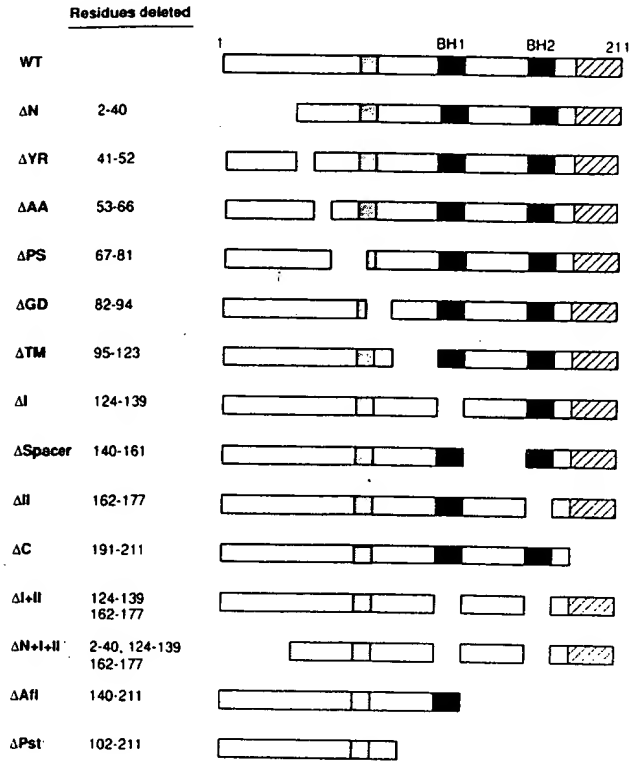


Fig. 1. Structures of Bak deletion mutants. The structures of wild-type (WT) Bak and Bak mutants are illustrated schematically. Black rectangles denote Bcl-2 homology domains 1 and 2; the cross-hatched boxes indicate the hydrophobic tail and the shaded boxes show the location of Bcl-2 homology domain 3 (amino acids 78–86, see text and Figure 4 for description). Numbers refer to the amino acid region(s) of Bak deleted in the corresponding mutant. All Bak mutants contained an amino-terminal HA epitope tag.

Results

Identification of Bak domains required for cell killing function

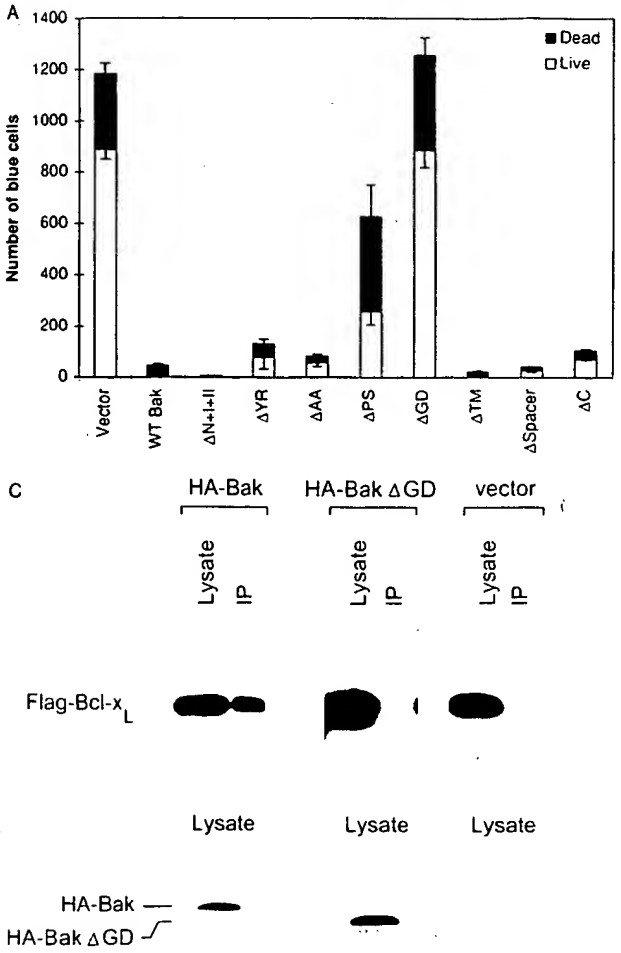
Previous experiments demonstrated that enforced Bak expression induces apoptosis in stably transfected Rat-1 cell lines (Chittenden *et al.*, 1995). To identify regions of the molecule that are necessary to induce apoptosis, a series of deletion mutants spanning the Bak protein were constructed (Figure 1) and tested for cell killing activity by a transient transfection assay in Rat-1 cells. This assay is similar to that previously described for detecting cell death induced by interleukin (IL)-1β-converting enzyme (Miura *et al.*, 1993; Kumar *et al.*, 1994; Wang *et al.*, 1994). Rat-1 cells were transfected with a marker plasmid encoding β-galactosidase (β-gal), in combination with either an expression plasmid encoding Bak, or various control plasmids. Transfected cells were stained with X-gal 24 h after transfection to detect β-gal-expressing cells. The cell killing activity of Bak in this assay is manifested by a large reduction in the number of blue (β-gal-expressing) cells obtained relative to co-transfection of the β-gal plasmid with a control expression vector (Figure 2A; compare WT Bak and Vector). The elimination of blue cells indicates that transfected cells are killed by Bak, an interpretation supported by the measurable reduction in total cell number in wells transfected with Bak expression plasmids (not shown). Furthermore, IL-1β-converting enzyme, previously shown by a similar assay to induce apoptosis in Rat-1 cells, also eliminated blue cells in this assay when expressed from the vector employed here (Boyd *et al.*, 1995). Control expression plasmids harboring the *bak* cDNA in the anti-sense orientation, or various unrelated cDNAs, did not eliminate β-gal-positive cells. In addition, the number of blue cells could be partially restored by co-transfection of Bak with Bcl-x_L or E1B 19 kDa (not shown), suggesting that the elimination of blue cells is a reflection of an apoptotic (Bcl-x_L-inhibitable) function of Bak.

Analysis of the Bak deletion mutants revealed that much of the Bak molecule is dispensable for its cell death function detected by this assay (Figure 2A; summarized in Table I). The non-essential regions include BH1 and BH2, the two domains in the carboxy-terminal half of the protein that show the highest degree of homology to other Bcl-2 family members. Deletion of the carboxy-terminal hydrophobic stretch of amino acids (residues 191–211; mutant ΔC) slightly diminished the cytotoxic function of Bak (Figure 2A). By analogy to the role of similar sequences in other Bcl-2 relatives (Tanaka *et al.*, 1993), this hydrophobic 'tail' probably serves as a membrane anchor sequence in Bak. While the carboxy-terminal hydrophobic tail is not required for the cell killing function of Bak, it may contribute to activity by ensuring proper subcellular localization of the protein.

A segment of the Bak protein removed by the ΔGD deletion (residues 82–94) is absolutely essential for cell death function, since this mutant exhibited no cell killing activity in the transient transfection assay (Figure 2A). Deletion of adjoining residues (amino acids 67–81) immediately N-terminal to this region also significantly reduced cell death activity (Bak mutant ΔPS). The loss of activity was not related to an obvious defect in protein expression since both ΔGD and ΔPS were expressed at levels similar to wild-type Bak in transfected cells (i.e. Figure 2C, and data not shown). All other deletion mutants tested were unaltered, or slightly diminished, in their capacity to kill Rat-1 cells (Figure 2A and Table I). Taken together, these results indicate that a co-linear segment, defined by the deletion mutants ΔGD and ΔPS (residues 67–94), is uniquely required for the cytotoxic function of Bak detected in the transient assay.

Identification of Bak sequences that mediate the interaction with Bcl-x_L

Physical interaction with other Bcl-2 family members, such as Bcl-x_L, may be essential for Bak to exert its cell death function, or may regulate its activity. We therefore sought to identify elements within Bak that are necessary for its Bcl-x_L binding activity. The interaction of Bak with Bcl-x_L was detected both by an *in vitro* protein binding assay, and by co-immunoprecipitation from transfected cells. *In vitro* translated ^{35}S-labeled Bak bound to a purified, bacterially expressed glutathione S-transferase (GST)–Bcl-x_L fusion protein (Figure 2B). The specificity of this *in vitro* interaction was demonstrated by the failure of Bak to bind to purified GST alone (Figure 2B) and by the failure of GST–Bcl-x_L to bind to non-relevant proteins such as luciferase, α-factor or β-lactamase (not shown). A specific Bak–Bcl-x_L interaction was also detected by co-transfecting epitope-tagged forms of Bak and Bcl-x_L into COS cells. Bak was immunoprecipitated from trans-



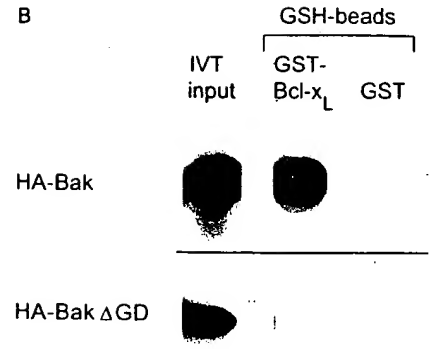


Fig. 2. Identification of a Bak domain required for cell killing and Bcl-x_L binding. (**A**) Detection of the cell killing activity of Bak in a transient transfection assay. Rat-1 cells were co-transfected with a β-gal marker plasmid and either a control plasmid (vector), or a plasmid expressing HA epitope-tagged Bak (WT Bak) or the indicated Bak deletion mutants (see Figure 1 for description of mutants). Cells were fixed and stained with X-gal 24 h after transfection, and the number of blue cells (β-gal-positive) counted by microscopic examination. The data shown are the number of dead (filled bars) and live (open bars) blue cells for each condition (mean ± SD from triplicate transfections). (**B**) Interaction of Bak with Bcl-x_L *in vitro*. *In vitro* translated ^{35}S-labeled HA-Bak or HA-Bak mutants (IVT input) were mixed either with GST–Bcl-x_L or GST. The complexes were captured on glutathione–agarose beads (GSH-beads), and bound ^{35}S-labeled protein was detected by SDS–PAGE and autoradiography. (**C**) Interaction of Bak with Bcl-x_L in transfected cells. COS cells were co-transfected with plasmids expressing Flag epitope-tagged Bcl-x_L (Flag-Bcl-x_L) and either HA-Bak, HA-Bak mutants or a control plasmid (vector). HA epitope-tagged proteins were immunoprecipitated (IP) from transfected cell lysates and associated Bcl-x_L was detected by immunoblotting with anti-Flag antibody (top panel). Expression of HA-tagged proteins was confirmed by immunoblotting with anti-HA antibody (bottom panel). The results for Bak and Bak ΔGD are shown; see Table I for summary of results for other mutants.

fected cell lysates and associated Bcl-x_L was detected by Western blot analysis of co-precipitated proteins (Figure 2C). Bcl-x_L was not detected in immunoprecipitates in the absence of co-expressed Bak, demonstrating that binding is specific.

The series of Bak deletion mutants described above (Figure 1) were tested for their Bcl-x_L binding capacity both *in vitro*, and in transfected COS cells (Figure 2B and C). Deletion of residues 82–94 (Bak mutant ΔGD) completely eliminated the ability of Bak to interact with Bcl-x_L. Interaction with Bcl-x_L was also greatly diminished by deletion of adjoining amino acids 67–81 (Bak mutant ΔPS). All other deletion mutants tested, encompassing the entire Bak open reading frame, retained the ability to bind Bcl-x_L (summarized in Table I). These results identify a contiguous Bak domain encompassed by the ΔGD and ΔPS mutants (maximally, amino acids 67–94) as uniquely important in mediating the interaction with Bcl-x_L. The same region of Bak was uniquely required for the cell killing function of Bak (see above).

A Bak domain is sufficient for cell killing activity and interaction with Bcl-x_L

The mutational analysis demonstrated the importance of Bak residues 67–94, however, it remained to be determined if this domain was itself sufficient for the biological activities of Bak. To explore this possibility, truncated Bak protein derivatives encompassing sequences within this region were tested for cytotoxic activity in Rat-1 cells and for their ability to bind Bcl-x_L (Figure 3). QVG (Bak amino acids 58–103; see Figure 3A) significantly reduced the number of blue cells when co-transfected with β-gal, indicating that it retained cell killing activity (Figure 3B). PEM (amino acids 73–123) showed little, if any, cytotoxic activity. However, PEM (and QVG) lacks the carboxy-terminal hydrophobic element and might exhibit reduced cell killing function due to altered subcellular localization. In an effort to improve the cell killing capacity of the truncated Bak species, the hydrophobic tail element (amino acids 187–211) was fused to the carboxy-termini of both PEM and QVG (to form PEM+C and QVG+C, respectively; Figure 3A). Attachment of the hydrophobic tail dramatically improved the ability of PEM to eliminate blue cells in the transfection assay, and resulted in cytotoxicity similar to wild-type Bak (Figure 3B). These results suggest that a protein domain shared by both PEM and QVG (amino acids 73–103) is sufficient for the cell killing function of Bak, in the context of the putative membrane anchor sequence.

We next examined whether QVG and PEM retained the ability to bind Bcl-x_L *in vitro* and in transfected cells (Figure 3C and D). *In vitro* translated QVG and PEM bound specifically to GST–Bcl-x_L and, in a reciprocal experiment, *in vitro* translated Bcl-x_L interacted with a

Table I. Summary of cell killing and protein binding activity of Bak deletion mutants

Bak mutant	Residues deleted	Cell killing activity	Bcl-x_L binding activity
WT	–	+	+
ΔN	2–40	+	+
ΔYR	41–52	+	+
ΔAA	53–66	+	+
ΔPS	67–81	±	±
ΔGD	82–94	–	–
ΔTM	95–123	+	+
ΔI	124–139	+	+
ΔSpacer	140–161	+	+
ΔII	162–177	+	+
ΔC	191–211	+	+
ΔI+II	124–139, 162–177	+	+
ΔN+I+II	2–40, 124–139, 162–177	+	+
ΔAfl	140–211	n.d.	(+)
ΔPst	102–211	n.d.	(+)

Cell killing activity (detected in the transient transfection assay) and Bcl-x_L binding activity (detected both *in vitro* and in transfected cells) of the Bak mutants relative to wild-type Bak is summarized as follows: +, equivalent or slightly diminished; ±, greatly diminished; –, defective. n.d. indicates experiment not done. (+) indicates binding was tested *in vitro* only.

GST–PEM fusion protein (Figure 3C). Both PEM and QVG were co-immunoprecipitated with Bcl-x_L from transfected cell extracts (Figure 3D), indicating that each contains a domain sufficient for mediating the interaction with Bcl-x_L. In addition, this domain was sufficient to mediate binding of the viral cell death suppressor, E1B 19 kDa, as shown by the specific interaction of *in vitro* translated E1B 19 kDa with GST–PEM (Figure 3C). Together with the analysis of the deletion mutants described above, these results demonstrate that amino acid sequences spanning residues 73–94 comprise a key functional domain in Bak that mediates cell killing and protein binding functions.

Sequences homologous to the Bak functional domain are present in Bax and Bip1 and are critical for their activities

Mutational analysis of Bak revealed that the same region, maximally residues 67–94, was uniquely involved in both the cell killing and Bcl-x_L binding activities of Bak. Two other Bcl-2 binding proteins, Bax and Bip1 (cloned by virtue of its interaction with Bcl-2; Boyd *et al.*, 1995), have biological activities that closely resemble those of Bak. Both Bax and Bip1 eliminate blue cells when co-transfected with β-gal in Rat-1 cells, indicating that they also are cytotoxic in this context. Bax and Bip1 also interact specifically with Bcl-x_L both *in vitro* and in transfected COS cells (Boyd *et al.*, 1995). These functional similarities prompted us to examine whether there were any structural features shared by the three proteins that contribute to their similar biological functions. Specifically, in light of the results described above, we examined whether there are sequences in Bax and Bip1 that resemble the functional domain in Bak (residues 67–94) and are likewise important for their biological activities.

Bax shows extensive homology to Bcl-2 family members (including Bak), with the highest degree of sequence homology centered around BH1 and BH2 (Oltvai *et al.*, 1993). A stretch of amino acids in Bax (residues 59–77) is homologous to sequences within the Bak functional domain (residues 74–94; Figure 4). In contrast to Bax, the primary sequence of Bip1 does not closely resemble the known Bcl-2 relatives and most notably lacks sequences matching BH1 and BH2, the defining elements of the Bcl-2 family (Boyd *et al.*, 1995). However, inspection of the Bip1 sequence identified a region (amino acids 57–69) that shows similarity to the same homologous element within Bak and Bax (Figure 4).

We tested if the homologous elements within Bax and Bip1 are also critical for the respective cell killing and protein binding functions of these proteins. Deletion mutations that removed the conserved sequence motifs were introduced into Bax and Bip1 (Figure 4), and the resulting mutants were analyzed for their ability to kill Rat-1 cells and to bind Bcl-x_L (Figure 5). Like Bak ΔGD, the Bax ΔGD and Bip1 ΔGD mutants were substantially impaired in their ability to eliminate blue cells when co-transfected with β-gal in Rat-1 cells (Figure 5A). In addition, both mutants had lost the capacity to interact with Bcl-x_L *in vitro* (Figure 5B) and no longer bound Bcl-2 in transfected COS cells (not shown). Thus, the structure of the Bak functional domain is conserved in Bak, Bax and Bip1, and is critical to the biological activities of all three proteins.

Discussion

A systematic structure/function analysis of Bak revealed that its ability to promote cell death and bind to Bcl-x_L is mediated primarily by a single domain located in the central portion of the Bak molecule. Small, truncated forms of Bak encompassing this region were cytotoxic to Rat-1 cells, and retained the capacity to bind Bcl-x_L, indicating that the domain is sufficient for these activities. Sequences homologous to a motif within the Bak functional domain are present in the central portions of Bax and Bip1, two other cell death-promoting proteins that interact with Bcl-x_L. The presence of a similar sequence in Bip1 is particularly noteworthy since, apart from this homology, the primary sequence of Bip1 does not overtly resemble that of Bak or Bax. Thus, all three proteins interact with Bcl-x_L, promote cell death and share this sequence motif. Mutations in the homologous sequences in Bax and Bip1 greatly diminished their cell death-promoting and protein binding functions, implicating the importance of this domain in the activity of diverse cell death regulatory proteins.

The conserved sequence elements within the Bak functional domain are not exclusively present in the cell death-promoting proteins Bak, Bax and Bip1. Alignment of Bcl-2 and Bcl-x_L with Bax and Bak has previously noted homologies within this region (residues 97–105 and 90–98 of Bcl-2 and Bcl-x_L, respectively) (Boise *et al.*, 1993; Oltvai *et al.*, 1993; Williams and Smith, 1993; Chittenden *et al.*, 1995). In view of the homology of these sequences among Bcl-2 family members, we propose to label this conserved region BH3 (see also Boyd *et al.*, 1995). The role of BH3 in anti-apoptotic proteins such as Bcl-2 and Bcl-x_L remains to be elucidated; however, it may also prove to be functionally important. Substitution of two

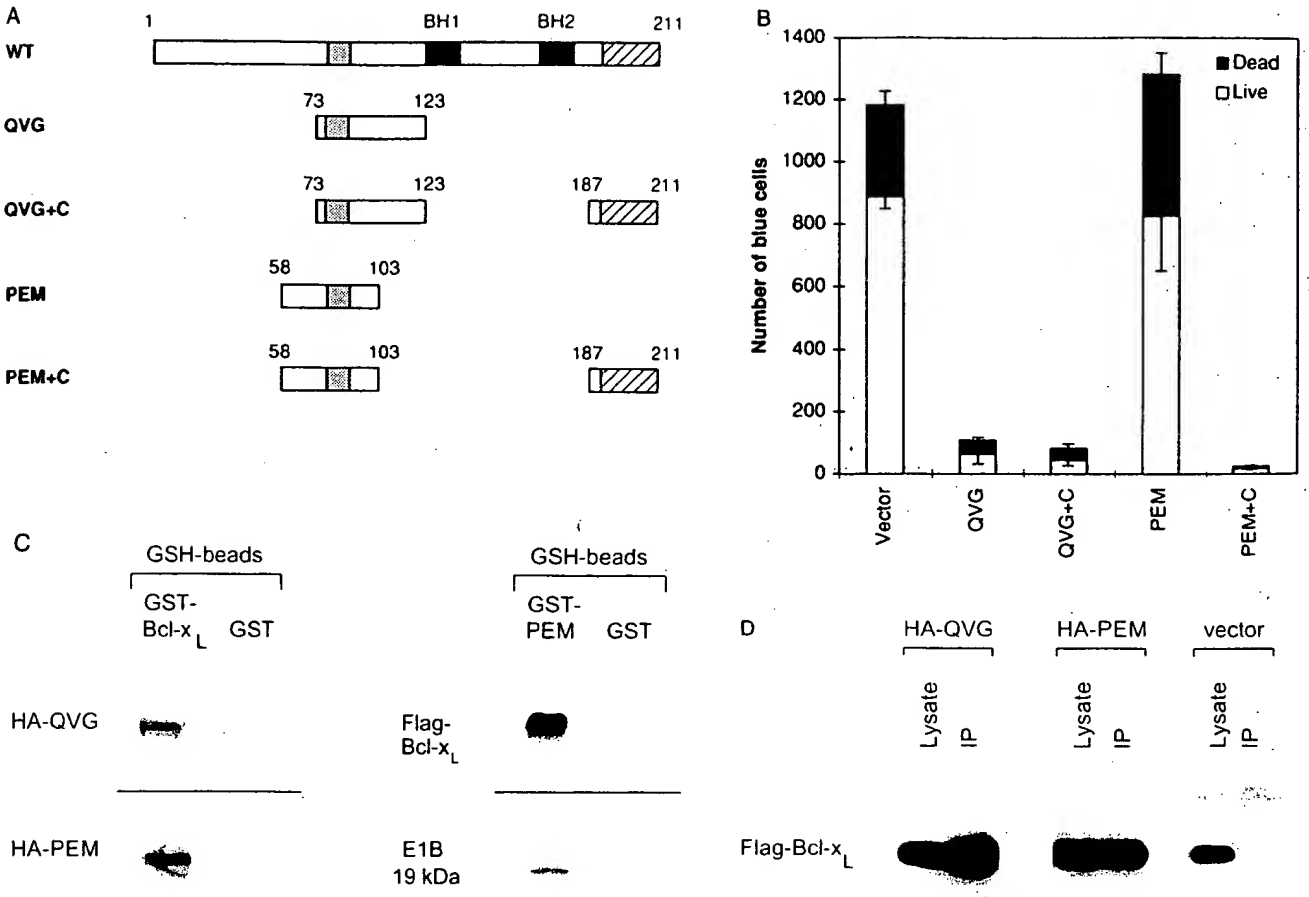


Fig. 3. A domain in the central portion of Bak is sufficient for cell killing and protein binding. (**A**) Schematic representation of truncated Bak derivatives. Bcl-2 homology domain 3 (see text and Figure 4) is shown as a shaded box; the hydrophobic tail is indicated by the cross-hatched rectangle. Numbers refer to the endpoints of Bak amino acids retained in the truncated species. All Bak derivatives contained an amino-terminal HA epitope tag. (**B**) Cell killing activity of truncated Bak derivatives in Rat-1 cells. Truncated Bak species with (QVG+C and PEM+C) or without (QVG and PEM) the carboxy-terminal hydrophobic tail were tested for cell killing activity by co-expression with β-gal in Rat-1 cells, as described in Figure 2A. (The vector control from Figure 2A is reproduced here since these data were all derived from the same transfection experiment.) (**C**) Interaction of truncated Bak derivatives with Bcl-x_L and E1B 19 kDa *in vitro*. Binding of *in vitro* translated ^{35}S-labeled QVG and PEM (HA epitope-tagged) to GST–Bcl-x_L (left panel) was assayed as described in Figure 2B. Interaction of *in vitro* translated ^{35}S-labeled Flag epitope-tagged Bcl-x_L and E1B 19 kDa with a GST fusion protein encoding residues 58–103 of Bak, GST–PEM (right panel), was assayed as described in Figure 2B for binding assays with GST–Bcl-x_L. (**D**) Interaction of truncated Bak derivatives with Bcl-x_L in transfected cells. The interaction of truncated Bak species (HA epitope-tagged) with Bcl-x_L (Flag epitope-tagged) was analyzed in transfected COS cells, as described in Figure 2C.

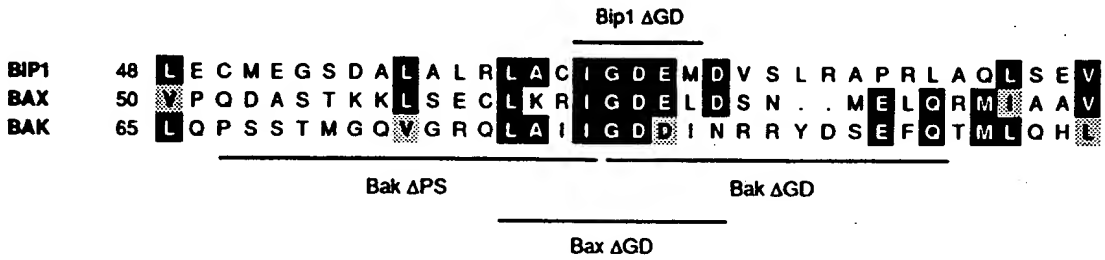


Fig. 4. Bip1 and Bax share sequences homologous to a motif within the functional domain of Bak. Numbers refer to the amino acid position of the portions of the Bip1, Bax and Bak sequences shown. Solid black shading indicates sequence identity between at least two of the proteins, and gray shading shows conservative amino acid substitutions. Solid lines delineate the amino acids deleted in the Bip1 ΔGD, Bax ΔGD, Bak ΔGD and Bak ΔPS mutants.

amino acids within the BH3 element of Bcl-2 (residues 97 and 98) abrogated the anti-apoptotic function of Bcl-2 (T.Subramanian and G.Chinnadurai, unpublished). Moreover, deletion of BH3 sequences from Bcl-x_L (GDE; amino acids 94–96) eliminated its ability to bind to Bak and suppress cell death in the assays described here (T.Chittenden, C.Flemington, R.G.Ebb and R.J.Lutz, unpublished results).

The regions of Bak that are the most conserved among Bcl-2 homologs, BH1 and BH2, were not required for cell death function or Bcl-x_L binding. Similarly, deletion of BH2 in Bax did not detectably alter its capacity to

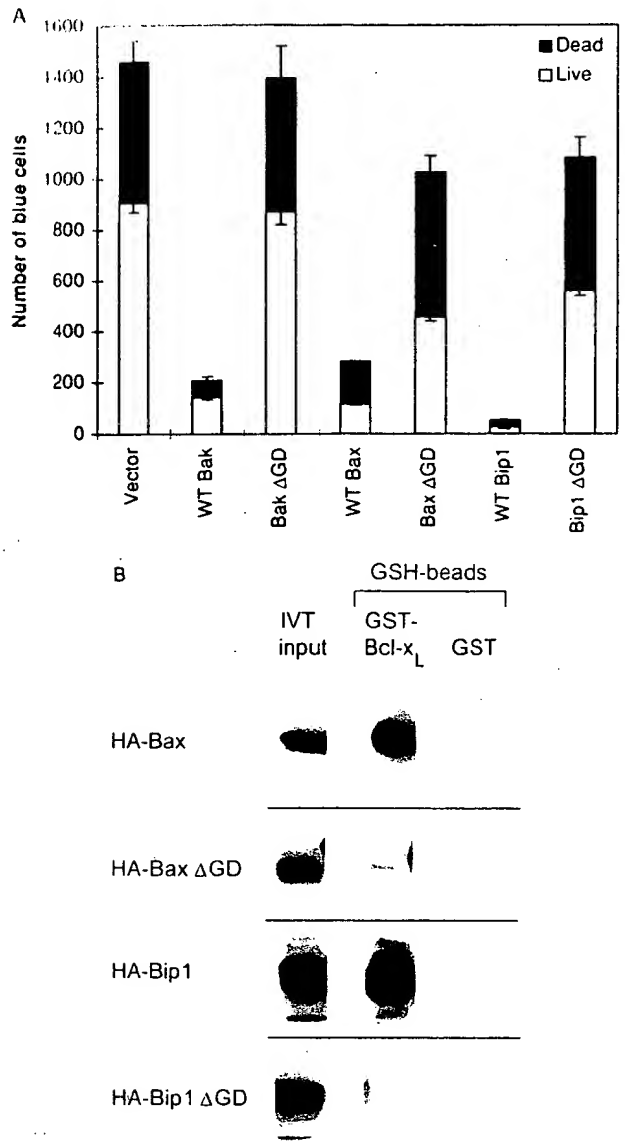


Fig. 5. The conserved domains in Bax and Bip1 are critical for their cell killing and protein binding functions. (**A**) Transient transfection of Bax and Bip1 mutants in Rat-1 cells. Cell killing activity of wild-type (WT) and ΔGD mutants of Bax and Bip1, upon co-expression with β-gal in Rat-1 cells, was detected as described in Figure 2A. Wild-type Bak and Bak ΔGD were included for comparison. (**B**) Interaction of Bax and Bip1 mutants with Bcl-x_L *in vitro*. ^{35}S-labeled HA epitope-tagged Bax, Bip1 and their respective ΔGD mutants were synthesized *in vitro* and assayed for specific binding to GST–Bcl-x_L as described in Figure 2B.

promote cell death or bind to Bcl-x_L (not shown), and Bip1 lacks canonical BH1 and BH2 domains. In contrast to these cell death promoters, Bcl-2 requires the integrity of both BH1 and BH2 to suppress apoptosis and bind to Bax (Yin *et al.*, 1994). It appears that BH1 and BH2 are similarly required for Bcl-x_L function, since Bcl-x_S, which lacks BH1 and BH2, no longer binds Bak or Bax and fails to suppress apoptosis (Boise *et al.*, 1993). These results point to significant differences between cell death promoters and cell death suppressors with respect to the sequence elements required for heterodimerization. For the death promoters (at least Bak, Bax and Bip1), sequences encompassing the BH3 domain appear to mediate the interaction. For the anti-apoptotic partner (i.e. Bcl-2) the requirements appear more complex and involve a combination of domains including BH1, BH2, BH3 and perhaps others (Boyd *et al.*, 1994; Sato *et al.*, 1994; Yin *et al.*, 1994).

While we have focused primarily on binding of Bak to Bcl-x_L in this study, this may not represent the only significant interaction governed by the BH3 domain. This region of Bak is required for interaction with Bcl-2 *in vitro* (not shown), and evidence presented here indicates that the BH3 domain will likewise mediate binding to E1B 19 kDa. It is therefore likely that other viral and cellular Bcl-2 homologs may also interact with Bak via its BH3 sequences. The apparent conservation of BH3 structure and function in Bak, Bax and Bip1 suggests that these proteins heterodimerize with anti-apoptotic proteins such as Bcl-x_L and E1B 19 kDa in a mechanistically similar fashion. However, the BH3 domain may not be the only domain involved in mediating protein interactions with Bak. While BH1 and BH2 were not required for the activities of Bak measured here, these conserved domains may contribute to the regulation of Bak or facilitate interactions with Bcl-2 homologs and/or as yet unidentified proteins. A role for BH1 and BH2 in facilitating cell killing and protein binding can also not be excluded, since the assays used in this study are unlikely to detect small differences in efficacy or affinity.

Thus far, the cell killing activity of Bak could not be separated clearly from its Bcl-x_L binding activity by the mutational analysis (see Table I). Bak mutants that lost cell killing activity (ΔGD and ΔPS) correspondingly lost Bcl-x_L binding activity, indicating that these two functions may be closely linked. One of the truncated Bak molecules tested, PEM, appeared to retain Bcl-x_L binding capacity but did not exhibit detectable cell killing activity. However, cytotoxic function was restored upon addition of the hydrophobic tail element to PEM (PEM+C), suggesting that the reduced cytotoxicity of PEM was related to its subcellular localization. This interpretation does not explain why the truncated Bak molecule QVG and the Bak mutant ΔC, which both lack the tail element, retained significant cell killing activity.

The link between Bcl-x_L binding and cell killing can be reconciled, in light of the available data, with Bak operating as either a regulator or as an active component of the cell death pathway. One possibility is that Bak may kill cells by binding, via its BH3 domain, and inhibiting molecules like Bcl-x_L that actively promote cell survival. Cell death would result from the suppression of essential survival functions provided by Bcl-x_L or related anti-apoptotic proteins. A second possibility is that Bak, Bax and Bip1 actively promote cell death, and are themselves suppressed by their interactions with Bcl-x_L, Bcl-2 and E1B 19 kDa. By this view, the BH3 domain comprises a critical cell death effector domain that may be masked by the binding of survival proteins like Bcl-x_L or E1B 19 kDa. Either scenario distinguishes the BH3 domain as an important molecular target and suggests that molecules developed to mimic or block the activity of this functional domain may prove to have interesting cell death regulatory properties and potential therapeutic utility.

Materials and methods

Plasmids and DNA manipulations

All recombinant DNA procedures were performed following standard protocols (Sambrook *et al.*, 1989). cDNA clones corresponding to the Bax and Bcl-x_L open reading frame were generated by PCR amplification using primers based on the published DNA sequences (Boise *et al.*, 1993; Oltvai *et al.*, 1993); cloning of Bip1 is described elsewhere (Boyd *et al.*, 1995). Deletions in the *bak*, *bax* and *bip1* cDNAs were introduced by single step or two-step PCR mutagenesis methods (White, 1993). The amino acids deleted in the various mutants are listed in the text (Figures 1 and 4). Bak mutants ΔAfl and ΔPst were generated by digestion of the Bak cDNA with *Afl*I and *Pst*I, respectively, and were used for *in vitro* translation reactions (see below). The truncated Bak species QVG and PEM were generated by PCR amplification of cDNA segments encoding Bak residues 73–123 and 58–103, respectively. In each case, a *Cla*I site was introduced just prior to the stop codon in the 3′ primer used for amplification. This resulted in the addition of a single aspartate residue at the carboxy-terminus of QVG, and the addition of isoleucine–aspartate at the carboxy-terminus of PEM. A segment encoding the hydrophobic tail portion of Bak (residues 187–211) was amplified by PCR, with a *Cla*I site introduced within the 5′ primer. The hydrophobic tail segment was ligated, in-frame, to the 3′ ends of QVG and PEM via the *Cla*I sites, to create QVG+C and PEM+C. The additional residue(s) introduced by the *Cla*I site was retained at the junction sites in these Bak derivatives.

The sequences of mutations and all segments of cDNA amplified by PCR were confirmed by DNA sequence analysis. The construction of the hemagglutinin (HA) epitope-tagged Bak was described previously (Chittenden *et al.*, 1995). All Bak deletion mutants and truncated species were similarly tagged at the amino-terminus with the HA epitope (Kolodziej and Young, 1991) and cloned into the cytomegalovirus (CMV) enhancer/promoter expression plasmids pcDNA-1/Amp, pRcCMV or pcDNA-3 (Invitrogen, Inc.). These plasmids were used for transfection experiments and for *in vitro* transcription/translation reactions (see below).

GST fusion proteins were generated by cloning the Bcl-x_L cDNA and the truncated Bak species, PEM, in-frame with GST in pGEX2TK (Kaelin *et al.*, 1992). The Flag epitope (Kodak) was introduced at the amino-terminus of Bcl-x_L with synthetic oligonucleotides and cloned into pcDNA-3. The E1B 19 kDa encoding plasmid used for *in vitro* translation was described previously (Boyd *et al.*, 1994).

Transient transfection assay

Transient transfection assays to detect cell killing activity were performed essentially as described previously (Miura *et al.*, 1993; Kumar *et al.*, 1994; Wang *et al.*, 1994). One day prior to transfection, Rat-1 cells were plated in 24-well dishes at a density of 3.5×10^4 cells/well. The following day, the cells were transfected with a marker plasmid encoding β-galactosidase (0.16 μg), in combination with an expression plasmid encoding Bak, or the vector alone (0.42 μg), by the Lipofectamine procedure (Life Technologies). At 24 h post-transfection, cells were fixed and stained with X-gal to detect β-galactosidase expression in cells that received plasmid DNA (Miura *et al.*, 1993). The number of blue cells was counted by microscopic examination and scored as either live (flat blue cells) or dead (round blue cells). The cell killing activity of Bak in this assay was manifested by a large reduction in the number of blue cells obtained relative to co-transfection of the β-gal plasmid with a control expression vector (see text).

Detection of protein–protein interactions in vitro

GST and GST fusion proteins were expressed in *Escherichia coli* and purified by affinity chromatography using glutathione–agarose (GSH-beads) (Smith and Johnson, 1988). [^{35}S]Methionine-labeled proteins were synthesized *in vitro* using a coupled transcription/translation system (Promega) in rabbit reticulocyte lysates following the supplier's protocol. *In vitro* translation reactions were pre-cleared by mixing with 20 μl of bovine serum albumin (BSA)-washed GSH–agarose beads (50% slurry) at 4°C for 1 h in 0.1 ml 10 mM HEPES buffer, pH 7.2 containing 0.25% NP-40, 142.5 mM NaCl, 5 mM $MgCl_2$ and 1 mM EGTA (NP-40 lysis buffer). The beads were removed by centrifugation and the cleared supernatants were incubated either with GST or GST fusion proteins (final concentration 1 μM) at 4°C for 1 h. Protein complexes were recovered by incubation for 1 h with an additional 20 μl of GSH–agarose beads. The beads were washed twice with NP-40 lysis buffer followed by two washes with the same buffer without NP-40. Proteins were eluted from the beads by incubation in SDS–PAGE sample buffer at 100°C for 5 min and loaded onto 4–20% SDS–polyacrylamide gels. Following electrophoresis, gels were fixed and incubated in a fluorography enhancing solution (Amplify; Amersham). The gels were dried and subjected to autoradiography at −70°C.

Detection of protein–protein interactions in transfected cells

COS cells were seeded in 35 mm wells at a density of 2.0×10^5 cells/well. The cells were transfected with expression plasmids the following day by the Lipofectamine procedure (Life Technologies). Bak derivatives contained the HA epitope at their amino-termini; Bcl-x_L was tagged at its amino-terminus with the Flag epitope (Flag; Kodak). At 24 h post-transfection, cells were washed with phosphate-buffered saline (PBS) and lysed in NP-40 lysis buffer supplemented with 1 mM phenylmethylsulfonyl fluoride, 1 mM pepstatin and 1 μg/ml leupeptin. The lysates were incubated with anti-HA antibody (12CA5, Boehringer Mannheim) for 1 h and 20 μl of BSA-washed protein A–agarose beads (50% slurry) for an additional hour. The beads were washed twice with NP-40 lysis buffer followed by two washes with buffer lacking NP-40. Proteins were eluted from the beads by incubation in SDS–PAGE sample buffer at 100°C for 5 min and loaded onto 14% SDS–polyacrylamide gels. Following electrophoresis, proteins were transferred to Immobilon-P membranes (Millipore) and the membranes were blocked by incubation for 1 h with a 1% milk solution in PBS. Primary antibody (1 μg/ml 12CA5, Boehringer Mannheim; 28 μg/ml DAKO-bcl-2, 124, DAKO; 10 μg/ml anti-FLAG M2, Kodak) was incubated with the membranes for 1 h, followed by secondary antibody (0.8 μg/ml horseradish peroxidase-conjugated goat anti-mouse IgG; Jackson Laboratory) for an additional 1 h. Proteins were detected by enhanced chemiluminesence (ECL, Amersham) as described by the supplier.

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Note added in proof

The name of the protein referred to as Bip1 here has been changed to Bik (Boyd *et al.*, 1995). The GenBank accession number for Bik is U34584.